Different but the same: DNA identification reveals a striking colour variability in a Mediterranean eolid sea slug specimen (Mollusca: Nudibranchia)

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Abstract: A peculiar eolid nudibranch showing an unknown chromatic array was found in a rocky bottom of Santa Maria al Bagno, in the Salento peninsula, Ionian Sea (Central Mediterranean Sea). This specimen, initially identified as Piseinotecus sp., was observed in situ and photographed while feeding and laying eggs close to individuals belonging to the Mediterranean Piseinotecus soussi. To assess the identity of this unexpected Piseinotecus ‘white morph’, a DNA identification approach was carried out using mitochondrial cytochrome c oxidase subunit I (COI), as it is the molecular marker mostly used to distinguish nudibranchs species. The molecular analysis unambiguously identified this specimen as Piseinotecus soussi and helped to shed lights on the striking intraspecific colour variability characterizing this rare species.

Keywords: Piseinotecidae, Piseinotecus soussi, Intraspecific variability, Ionian Sea, Integrative taxonomy

INTRODUCTION

Nudibranchia is a group of Gastropoda molluscs known for their brilliant and diversified colours. The latter are linked to characteristic defensive strategies that nudibranchs have evolved together with the loss of the shell in the adult stage. In fact, these sea slugs lose the protective shell after metamorphosis entrusting their protection to different systems like the ability to accumulate toxic or repellent bioactive compounds, the adoption of peculiar behaviours and being cryptic with the substrate they live on. Therefore, the colour in these organisms is particularly important and has always been considered a useful diagnostic character for recognizing different species. Body colour in nudibranchs has a strong link with species evolutionary history and can influence species fitness, although it was found to be misleading in some cases (Furfaro and Mariottini, 2016; Furfaro et al., 2021). The genus Piseinotecus Er. Marcus, 1955 has an Atlantic and Mediterranean distribution with only one species, P. gonja Edmunds, 1970 described from the western Indian Ocean (Tanzania). This genus is represented by the Brazilian P. divae Er. Marcus, 1955 (as the type species) and nowadays includes six accepted species: P. ernestina Ortea & Moro, 2020 from Cape Verde, the previously mentioned P. gonja, P. minipapilla Edmunds, 2015 from Ghana, P. soussi Tamsouri, Carmona, Moukrim and Cervera, 2014 and P. sphaeripherus (Schmekel, 1965), which has also been reported from the Mediterranean Sea. Unlike the latter, which has been recorded only a few times from
the Mediterranean Sea (Zenetos et al., 2016; Salvador et al., 2022) without collecting specimens or depositing molecular data, P. soussi was morphologically and molecularly investigated and its geographical distribution (from the eastern Atlantic Ocean to the Mediterranean Sea) confirmed (Tamsouri et al., 2014; Furfaro and Mariottini, 2019; Furfaro et al. 2020). Interestingly, this small aeolid is phenotypically very similar to both Edmundsella albomaculata (Pola, Carmona, Calado & Cervera, 2014), a flabellinid known only from São Vicente Island (Cape Verde), and E. pedata (Montagu, 1816), a very common Mediterranean flabellinid. Records of P. soussi in the Mediterranean basin are relatively few, most probably due to its small size, elusive behaviour (Furfaro and Mariottini 2019; Salvador et al., 2022), and possible misidentification with E. pedata, as already mentioned by Furfaro and Mariottini (2019).

The area of Santa Maria al Bagno, (Lecce, Italy), located in the Ionian Sea, is one of the four Mediterranean sampling localities where P. soussi occurs (Tamsouri et al., 2014; Furfaro et al., 2020). This geographical spot encompasses a coastline of about 10 km, strongly influenced by the presence of karst cavities that have favoured the development of a very rich infralittoral biocenosis (Belmonte et al., 2010; Onorato and Belmonte, 2017). In this coastal stretch, wide cavities provide sciaphilic habitats that represent the ideal environment to favour the spreading of this taxon. Piseinotecus soussi has been annually observed during its breeding period, which in this Ionian area, due to its climatic conditions, takes place between the end of the winter and the beginning of spring (Furfaro et al., 2020). This aeolid is not very vagile and in Santa Caterina area it is common to observe it during mating or egg laying (Furfaro et al., 2020). During last year’s underwater observations, the authors have recorded the presence of a peculiar specimen, showing a strikingly different body colour pattern, among P. soussi individuals. This individual opened questions about the possible occurrence of unknown diversity or the presence of a high chromatic variability within P. soussi. Therefore, we aimed to i) investigate the genetic identity of the ‘white morph’ specimen using DNA identification analysis, ii) unravel the different chromatic patterns that can occur during the development of P. soussi and iii) present an iconography of a striking colour phenotype.

**MATERIALS AND METHODS**

*In situ* observations and sampling were carried out by scuba diving in Santa Maria al Bagno, in the Salento peninsula (Lecce, Apulia, Italy; 40.1316282, 17.9963146; Fig. 1), at 5 m depth during the spring of 2019. The nudibranch was photographed underwater on 27th of April 2019, then collected and observed in the laboratory, preserved in 95% ethanol, and finally deposited with voucher RM3_2262 in the collection of the Department of Science of the Roma Tre University. Photos of the specimens and of the diagnostic characters (like the shape of the rhinophores, distribution and shape of the cerata, body colour pattern) were taken in daytime dive using specific equipment dedicated to underwater macro photography (Nikon D7000 with 60 mm micro Nikkor lens, with Isotta case and two Inon Z240 flashes).

Total genomic DNA was extracted from one individual by selecting a small piece of foot tissue and by using the ‘salting out’ procedure (Aljanabi and Martinez, 1997). The primer pair LCO1490 and HCO2198 (Folmer et al., 1994) was used for the amplification of the mitochondrial cytochrome oxidase subunit I (COI) gene, with following cycling parameters: 5 min of initial DNA denaturation at 94 °C; 35 cycles of 94 °C/30 s (DNA denaturation), 48 °C/60 s (annealing), 72 °C/60 s (elongation); and 7 min of final extension at 72 °C (Furfaro et al., 2016). The final volume of the PCR reaction was 20 µl. The amplified product was sequenced at the European Division of Macrogen Inc. (Amsterdam, The Netherlands). The COI sequence was edited with Staden Package 2.0.0b9 (Staden et al., 1990). BLASTN (Altschul et al., 1990) search was conducted in the GenBank database to confirm the identity of the sequenced specimens.
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Fragment and to exclude contaminations. Consensus sequence was aligned together with GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) sequence using the Muscle algorithm implemented in MEGA 6.0 (Tamura et al., 2013). Since the COI mitochondrial marker is the most commonly used for species barcoding in Heterobranchia, our analysed data set consisted of our one sequence and six sequences obtained from GenBank (Table 1). The number of COI base differences per site from averaging over the sequence pair was calculated, and the mean uncorrected p-distances were obtained using MEGA 6.0 software (Tamura et al., 2013).

RESULTS

During field observations, specimens of P. soussi could be found (from February to June up to 6-8 individuals could be found per dive) crawling on hard substrates covered by red algae and epiphytic hydrozoans. In young individuals, the notum was transparent so that the major internal organs, especially the reproductive organs, the hepato-pancreatic endings in the cerata and the cnidosacs, were visible (Fig. 2). In subadult and adult individuals, the red colour of the cerata with the typical white speckling, the whitish cnidosacs, the bluish colour of the base of the rhinophores and the white speckling of their terminal half, as well as the basal blue-purple and the white spotted apical part of cephalic tentacles, were clearly visible (Fig. 2). Among them, a single individual showed a different chromatic pattern, almost completely lacking any blue-violet coloration (Fig. 3). This specimen, here defined as a ‘white morph’, was recorded together with the typical P. soussi morphotypes here defined as ‘typical morphs’ (Furfaro et al., 2018; Furfaro and Mariottini, 2019). This adult individual, of about 10 mm in length, displayed nine rows of red orange cerata, densely spotted with white dots, notum, rhinophores and cephalic tentacles with white evident speckles (Fig. 3). Furthermore, the cerata apical part contained the cnidosacs, which were clearly visible through the transparent epithelium and which had a typical white ring in the upper part (Fig. 4A). The white pigment that covered the whole body reached the cephalic portion where the faint purple-violet masticatory plates were visible due to transparency (Fig. 4C).

Molecular analysis

DNA extraction and sequencing allowed to obtain a COI sequence 605 bp long (COI Accession Number: OQ921890). The results from the pairwise comparison revealed a range of 0.5% and 0.8% of uncorrected COI p-distances that are shown in Table 2 and confirmed the ‘white morph’ identification as P. soussi, even though it had an unusual body colour pattern.

Table 1. Collection locality, voucher, COI accession number from GenBank and reference of Piseinotecus soussi specimens here analysed.

<table>
<thead>
<tr>
<th>Piseinotecus soussi</th>
<th>Locality</th>
<th>Voucher</th>
<th>COI Accession Number</th>
<th>References</th>
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<td>‘Typical morph’</td>
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<td>RM3_862</td>
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<td></td>
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Fig. 2. Young (A), subadult (B) and adult (C, D) individuals of Piseinotecus soussi photographed in laboratory and in situ from Santa Maria al Bagno (Lecce, Italy, Ionian Sea). The typical egg ribbon (white arrow) is visible in (C). Scale bars = 5 mm.
DISCUSSION

The DNA identification method is considered a powerful tool for detecting hidden diversity and/or determining the ranges of intraspecific morphological variability within species. Here we report the case of DNA identification technique applied to a peculiar eolid nudibranch observed in Santa Maria al Bagno, in the Salento peninsula (Ionian Sea), which was initially identified as *Piseinotecus* sp. This specimen, named ‘white morph’ as the consequence of its overall white body, was found with *P. soussi* specimens, a rare species with a wide geographical range but poorly recorded in the Mediterranean Sea (Furfaro and Mariottini, 2019). To understand if the ‘white morph’ was conspecific with *P. soussi* or if it was perhaps a new Mediterranean species, DNA comparison was carried out using the COI mitochondrial molecular marker, the most used in nudibranchs. The COI sequence obtained from the ‘white morph’ specimen was compared with the ones already available in GenBank and belonging to *P. soussi*, revealing a range of *p*-distance between 0.5% and 0.8%, that is within the range of intraspecific variability widely

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accepted for Nudibranchia molluscs (Furfaro et al., 2018; Furfaro and Mariottini 2021; Furfaro et al., 2021; Furfaro et al., 2022). Therefore, the obtained specimen was molecularly identified as P. soussi with distinctive body coloration, demonstrating that the combination of morphological observation and DNA identification is the best approach to resolve difficult taxonomic issues and to avoid misidentifications. This is particularly true in the case of Heterobranchia species, which are known to be morphologically very plastic in some cases (De Oliveira Saad et al., 2014). It is noteworthy to remind that P. soussi is apparently very similar to both Edmundsella albamucula and E. pedata, so that it could be of particular interest to deepen the study of the chromatic pattern also in these two Edmundsella species, in order to assess if there are some rare and hidden atypical morphotypes also in these pink eolids. Finally, this study confirms the importance of avoiding taxonomic decisions based only on morphology and re-evaluating them by considering additional evidence using different approaches such as molecular, ecological, and chemical.

**AUTHOR’S CONTRIBUTION**

G.F., F.V., P.M., Conceptualization; G.F., F.V., C.L., A.T., Data curation; F.V., C.L., Formal analysis; F.V., C.L., Investigation; G.F., F.V., C.L., P.M., Methodology; P.M., Supervision; G.F., F.V., C.L., A.T., P.M. Roles/Writing - original draft; G.F., A.T., P.M., Writing - review & editing

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